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Award Number: DAMD17-01-1-0667

TITLE: Use of ErbB-2 Mimotopes for Breast Cancer Immunotherapy

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REPORT DATE: July 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Final (15 Jun 2001 - 14 Jun 2003)

4. TITLE AND SUBTITLE
Use of ErbB-2 Mimotopes for Breast Cancer Immunotherapy

5. FUNDING NUMBERS
DAMD17-01-1-0667

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Recently a new drug (Herceptin) has been shown to be effective in the treatment of breast cancer, and has been approved for use. Herceptin is essentially a humanized mouse monoclonal antibody that binds to ErbB-2, a membrane growth factor receptor tyrosine kinase that is over expressed in 25-30% of patients with breast cancer. Herceptin prevents targeted cells from proliferating. Our goal is to induce the body to produce its own antibodies to ErbB-2, which hopefully like Herceptin will target breast cancer cells and then prevent them from growing. The specific purpose of this proposal was to test the use of phage particle mimetopes of ErbB-2, which when injected into mice, would induce the animals to make antibody against ErbB-2. Accordingly, we developed a phage selection protocol, and subsequently collected several M13 phage clones displaying high affinity binding to Herceptin. The selected clones were then used as immunogens to immunize Balb/c mice. At least two of these clones produced antibody responses that appear to be specific to ErbB-2 expressed on SKVO-3 cells. We have concluded that these mimotope phage clones are candidate immunogens for active immunotherapy trials in Balb/c mice with transplanted ErbB-2-positive tumors.

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
Herceptin, ErbB-2, phage mimotope

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

One of the major difficulties in using immunotherapy as an effective cancer treatment protocol has been to define suitable antigens to which immunotherapeutic protocols might be targeted. In the case of breast cancer, an excellent target for immunotherapy was identified over a decade ago. The HER2/neu proto-oncogene codes for a membrane receptor tyrosine kinase (ErbB-2) of the epidermal growth factor receptor family. The overexpression of ErbB-2 in ovarian, prostate and breast cancer makes it an attractive target for tumor immunotherapy [1,2]. Herceptin, a recombinant humanized monoclonal antibody with high affinity to ErbB-2, inhibits the growth of these tumor cells and is currently being used as a safe, effective reagent in combination with chemotherapy in women with HER-2-overexpressing metastatic breast cancer [3]. Unfortunately, this clinical application is an example of a passive immunotherapeutic approach, and, like in other passive immunotherapies, there are drawbacks. Some women do not achieve adequate serum levels of the reagent, while others in which Herceptin is initially effective, may develop an immunological defense reaction that neutralizes the drug. Clinical experience has shown that, although initial success is often achieved with passive immunotherapy, in the long run, active immunization is the superior treatment strategy. In this case, antibodies and T cells reactive to ErbB-2 have been isolated from breast cancer patients, indicating that ErbB-2 is a potential target for active immunization[4]. The aim of this research project was to evaluate the use of M13 phage particles displaying ErbB-2 (Herceptin reactive) mimotopes as immunization agents in mice. The goal was to stimulate the active production of antibodies that specifically recognize ErbB-2 at Herceptin defined sites in BALB/c mice. Phage display peptides (called mimotopes) isolated by immunoaffinity selection (biopanning) from linear or circular phage display libraries have been shown to induce antigen-specific immune responses directed against the epitope recognized by the monoclonal antibody used for the biopanning of the phage clones[5,6]. Mimotope immunization is therefore a way to induce epitope-specific antibody responses in vivo[7,8] for cases where use of the complete antigen is not desired.

Body

A. Research accomplishments associated with tasks outlined in the Statement of Work - *First Year (July, 2001- July, 2002):*

1. Isolated several M13 peptide display phage clones.

The Ph.D.-7 Phage Display Peptide Library kit (New England BioLabs) was used in these immunoaffinity selection experiments. This is a combinatorial library of random peptide 7-mers fused to a minor coat protein (pIII) of M13 phage. The library consists of ~2.8 x 10^9 electroporated sequences, amplified once to yield ~70 copies of each sequence in a 10µl phage sample used in each panning experiment. A panning protocol using elements of those published in the Ph.D.-7 kit instruction manual [9], plus modifications taken from 2 publications [10,11] was developed and used to immunoselect peptide display phage clones binding to Herceptin coating the affinity selection plates (Figure 1).

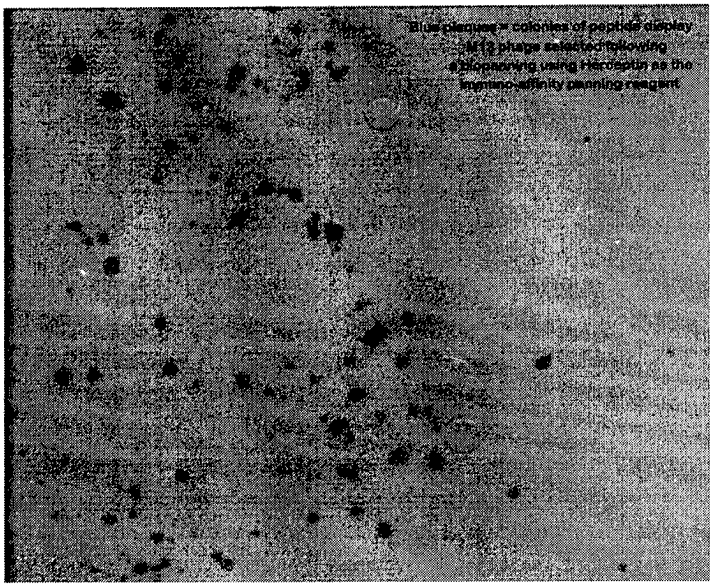

Figure 1. Phage clones isolated following biopanning with Herceptin. M13 is a male specific coliphage. It is propagated on *E coli* host strain ER2738, a rapid growth F+, lacZa- bacterium. The phage carries the lacZa gene, so plaques (bacterial colonies infected with a selected phage particle and its clones) appear blue when plated on this strain of *E coli* growing on media containing Xgal and IPTG.

2. Developed an effective protocol to generate high-titer phage clone solutions to be used as immunization reagents in mice. Generated several high-titer, amplified panning output (isolation) solutions that were stabilized in glycerol and stored at -23^{0}C.

Biopanning is a relatively easy, although time-consuming procedure. However, amplification of selected phage particles and production of a final reagent solution that had a high viral titer, yet no live *E coli* host bacteria (which would prove fatal to the test mice), proved to be more problem-prone than indicated in the published protocols. Again, published procedures were modified, and improved upon. The result was a reproducible, 1 day phage amplification protocol that routinely yielded 10^{15} to 10^{25} phage particles per milliliter of final solution. This final panning output solution usually has a total protein amount of 1 - 2 mg/ml and is almost always bacteria-free (all viable *E coli* viral host bacteria are removed from the final solution). Final isolated phage solutions collected and stored following Herceptin biopanning procedures are listed in Table 1.

Designation	phage PFU/ml	*E coli* CFU/ml	Designation	phage PFU/ml	*E coli* CFU/ml
4-12D-1-b	10^{15}	0	4-12D-2-b	10^{20}	100
4-17D-1	10^{20}	0	4-17D-2	10^{20}	10
4-24D-1	10^{16}	0	4-24D-2	10^{14}	0
5-1D-1	10^{19}	0	5-1D-2	10^{20}	0
7-9D-1	10^{20}	0	7-9D-2	10^{19}	0
5-1A	10^{20}	0	6-18A	10^{12}	10^{5}
5-8A	10^{22}	0	6-26A	10^{20}	0
5-15A	10^{22}	250			

Table 1 Phage containing solutions isolated from Ph.D.-7 Phage Display unconstrained 7-peptide library (New England BioLabs) by biopanning against Herceptin during the first year.

3. Gauged the Herceptin binding specificity of the phage solutions generated and stored by direct immunoblot affinity testing.

Although biopanning has a reputation in the literature of being highly selective for specific mimic peptides [12,13,14], we decided to directly gauge this specificity in our phage output solutions. 1 µl aliquots of stored phage and dilutions of these solutions were directly blotted to nitrocellulose in a series of experiments. After blocking with 1% BSA (Sigma), blots were probed with Herceptin as the primary detection antibody. Herceptin binding was determined by probing with a secondary antibody, goat anti-human Ig with HRP conjugate (SouthernBiotech). HRP on immunoblots was visualized with SuperSignal West Pico chemiluminescent substrate (Pierce). An example of one of these experiments is shown in Figure 2.

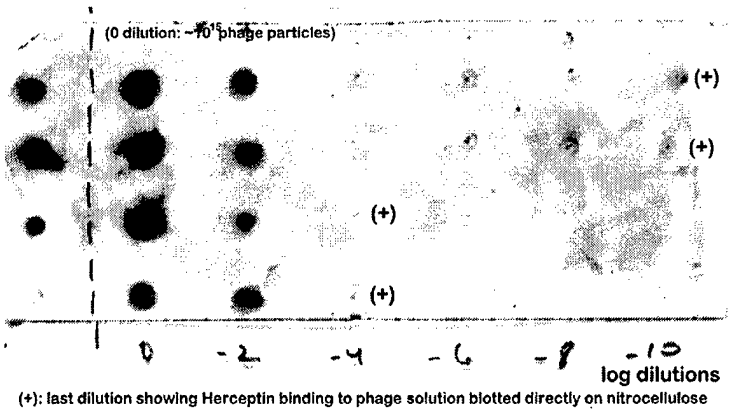

Figure 2. Direct immunoblots of 4 panning output phage solutions. Herceptin is the affinity antibody used to probe the phage blots. Dilutions of isolated mimotope phage solutions are shown blotted from the dotted line to the far right, while control blots are at the far left of the dotted line. Note that at higher concentrations, Herceptin is detected binding nonspecifically to M13 phage, as well as to both the wild type and the Ph.D. 7-peptide mimotope phage libraries used in the earlier biopanning experiments.

These experiments confirmed our earlier assumption that there might be a fair amount of nonspecific low affinity phage binding to Herceptin. However, it was also shown that some of the panned phage isolates collected have detectable Herceptin -affinity at high dilutions, suggesting that at least some of the phage in these isolations carried immuno-specific mimotopes to the Herceptin ErbB-2 binding site. However it was also possible that non-specific, but nevertheless high affinity, phage binding to Herceptin at sites other than the antibody combing site were also present. We felt that the problem of this type of nonspecific phage in the final biopanning output/isolation solutions had to be addressed before mice vaccination with these solutions could begin.

B. Research accomplishments associated with tasks outlined in the Statement of Work - *Second Year (July, 2002- July, 2003):*

1. Developed and added a phage library "human IgG pre-panning absorption" procedure to the biopanning experimental protocol that was developed and used in the first year.

To reduce the number of irrelevant mimotope display phage retained during the biopanning immuno-selection protocol, we added a "pre-panning" immunoabsorption procedure, modified from biopanning preparatory procedures done by others (15,16) to remove as many nonspecific-human IgG binding antigens from the phage libraries as possible. Ph.D.-7 ("A" panning outputs in Table 2) and Ph.D.C-7-C ("B" panning outputs in Table 2) random peptide display phage libraries were incubated with human IgG-agarose (Sigma) overnight before biopanning against Herceptin. Phage absorbed to the general mix of human antibodies on the agarose were discarded, and only unbound library phage solutions were amplified and used in biopanning experiments that followed. This pre-panning IgG absorption procedure was done twice and the absorbed phage library outputs panned against 2 different lots of Herceptin, producing 4 final phage isolation/output solutions designated 3-27A, 3-27B, 4-14A and 4-14B. The "A" designation indicates display phage selected by using Herceptin to biopan the absorbed, unconstrained peptide library, while "B" indicates display phage selected by using Herceptin to biopan the absorbed, constrained peptide library. Five isolated phage plaques from each of the 4 panned output phage titration cultures were randomly selected, amplified and titered. This yielded 20 single-mimotope phage clone solutions, each one picked from single plaques growing out of Herceptin-immunoaffinity selected peptide display phage cultures in *E coli.* The result was 24 immuno-selected, high-titer mimotope-display phage solutions, all possible immunization candidates for production of ErbB-2-specific antibodies in mice. These phage solutions are listed in Table 2.

Designation	phage PFU/ml	*E coli* CFU/ml	Designation	phage PFU/ml	*E coli* CFU/ml
3-27A-3rd	10^{20}	0	3-27B-3rd *(3)*	10^{20}	0
4-1A-1	10^{22}	0	4-1B-1 *(2)*	10^{22}	0
4-1A-2	10^{23}	0	4-1B-2	10^{22}	0
4-1A-3	10^{15}	0	4-1B-3	10^{22}	0
4-1A-4	10^{21}	0	4-1B-4	10^{21}	0
4-1A-5 *(1)*	10^{22}	0	4-1B-5	10^{21}	0
4-14A-3rd *(5)*	10^{17}	0	4-14B-3rd *(7)*	10^{17}	0
4-14A-1	10^{28}	0	4-14B-1	10^{26}	0
4-14A-2	10^{25}	0	4-14B-2	10^{26}	0
4-14A-3	10^{20}	0	4-14B-3	10^{26}	0
4-14A-4 *(4)*	10^{26}	0	4-14B-4 *(6)*	10^{26}	0
4-14A-5	10^{22}	0	4-14B-5	10^{25}	0

Table 2 Phage-containing solutions isolated from Ph.D.-7 Phage Display unconstrained 7-peptide library (designated as A above) and Ph.D.C-7-C 7-peptide constrained library (designated as B above) (New England BioLabs) by biopanning with Herceptin or by picking and amplifying single phage plaques obtained in culture procedures following biopanning during the second year. Both random mimotope display libraries were absorbed with human IgG-agarose to remove nonspecific IgG-binding mimotope phage before biopanning with Herceptin. *(#)* indicates solutions used as vaccination reagents in mice.

2. Gauged the Herceptin binding specificity of the IgG-absorbed and Herceptin-biopanned phage solutions generated by direct immunoblot affinity testing.

1- 4 µl aliquots of stored phage isolation solutions were directly blotted to nitrocellulose. After blocking with 1% sterilized milk/PBS, blots were probed with Herceptin as the primary detection antibody. Herceptin binding was determined by probing with a secondary antibody, goat anti-human Ig with HRP conjugate (SouthernBiotech). HRP on immunoblots was visualized with SuperSignal West Pico chemiluminescent substrate (Pierce). An example of one of these experiments is shown in Figure 3.

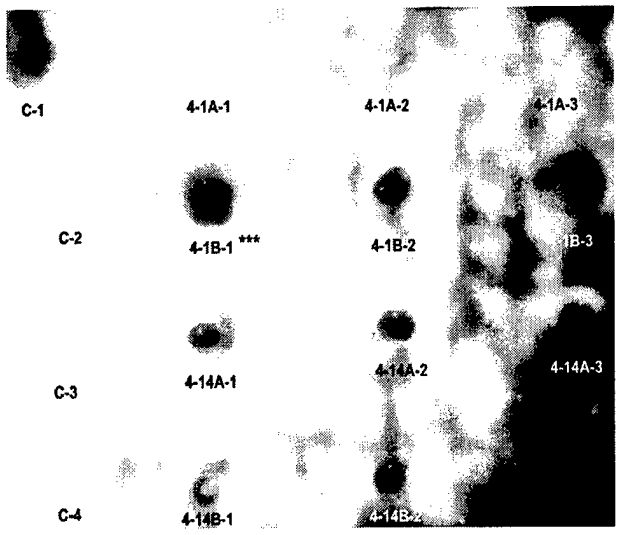

Figure 3 Direct immunoblots of selected panning output phage solutions (complete listing in Table 2). Herceptin is the affinity antibody used to probe the phage blots. Control blots are at the far left, designated as C-1 (wild type M13 phage amplification), C-2 (Ig-G-absorbed Ph.D.-7 unconstrained phage library amplification), C-3 (Ig-G-absorbed Ph.D.-C-7-C constrained library amplification and C-4 (no phage, *E coli*-only amplification solution). Note that Herceptin binds nonspecifically to wild type M13 phage (C-1), but not as strongly as it does to some of the immuno-selected phage mimotope isolates. C-1 and C-4 were both used as control immunogens (numbers *8* and *9*) in the mouse immunization trials that followed, as was the selected display phage clone indicated by the *** above (number *2*).

Herceptin showed a high affinity for binding to phage in most of the 4-14 output/isolation solutions, regardless of whether or not the mimotope(s) selected came from an unconstrained or constrained peptide-display library. The 3-27 output/isolations showed a much greater variability in ability to specifically bind Herceptin. Based on these tests, 7 phage output/isolation solutions were chosen for immunization. These are numbered *1* - *7* in Table 2 and include four of the picked, single mimotope phage clone cultures, plus the 3 phage panning output solutions that these specific clones were isolated from.

3. Used mimotope phage isolation solutions generated by Herceptin biopanning as immunization reagents in Balb/c female mice.

36 six-week old female Balb/c mice (Charles River Co.) were separated into nine groups of 4 and housed in separate cages. They were given a regular diet of mouse chow (Purina), chopped apples and water. The immunization protocol used was a modification of work done by other groups working with mimotope phage vaccines [7,17,18]. All mice within a group received a 150 µl intraperitoneal injection of the same selected phage output/isolation solution mixed with an equivalent amount of Freund's complete adjuvant (Sigma) on day 1. Groups 1 through 7

received the selected mimotope phage solutions, each group getting a phage vaccine solution with the same number designation. Groups 8 and 9 were controls: Group 8 receiving a M13 wild-type phage amplification/output solution and Group 9 getting the *E coli* amplification/output solution produced without phage. 150 µl booster IP injections consisting of the same phage solutions, but mixed with an equivalent amount of incomplete Freund's adjuvant were administered on day 8. All the mice were listless for a few days after the second inoculation, but all appeared to recover a week later. Cardiac blood was collected from all of the mice on day 31. Sera were separated and stored frozen (-23°C) for later testing.

4. Developed and used a competitive inhibition assay to screen mouse sera for the presence of antibodies that would specifically recognize and bind to ErbB-2 at Herceptin defined sites.

We initially proposed using ErbB-2 protein and ELISA to screen mouse sera and determine anti-ErbB-2 titers. However we decided that a competitive inhibition assay between antibodies in the sera and Herceptin for the ErbB-2 naturally displayed on human SKOV-3 tumor cell membranes would be a better test for epitope specificity of antibody generated by the phage mimotope immunogens. Consequently, aliquots of cultured SKOV-3 cells (ATCC, Rockville MD) were incubated first with dilutions of the selected sera, then with FITC-labeled Herceptin. After washing, simple wet mounts of each sample were prepared on regular microscope slides, and cells were imaged using an epifluorescence microscope (Nikon). This assay has tentatively identified two sera samples, from vaccine groups 3 and 4, as containing antibodies that appeared to inhibit and/or block labeled Herceptin from binding to ErbB-2 on SKOV-3 cells.

Key Research Accomplishments

1. Isolated several different M13 peptide display phage clones (mimotopes) from human IgG-absorbed random peptide display phage libraries using Herceptin in immuno- selective biopanning assays.

2. Developed an effective protocol to generate high-titer, *E coli*-free phage clone solutions to be used as immunization reagents in mice.

3. Developed and used a direct immunoblot assay to gauge the Herceptin binding specificity of mimotope phage solutions generated as immunization reagents. All mimotope phage solutions selected for use as immunization reagents in mice were bound by Herceptin with high affinity in these tests.

4. Competitive inhibition screening assays indicate that 2 test groups of Balb/c mice were immunized successfully by IP injections of 2 different, immuno- selected phage mimotope solutions to produce antibodies specific to the Herceptin binding site on ErbB-2.

Reportable Outcomes

None as of the writing of this report.

Conclusions

As of the end of the first year, we had generated several high titer, M13 peptide mimotope phage solutions using published biopanning protocols and Herceptin as the immuno-affinity selection agent. We had also developed a fairly effective amplification protocol to produce high-titer, bacteria-free immunogens from the selected phage solutions. But, based on direct immunoblot testing of these potential vaccines, mice immunization was delayed until the problem of nonspecific mimotope display phage present in the final biopanned output phage solutions was addressed. In the second year, we solved this problem by performing an immunoabsorption procedure with human IgG-agarose on the random peptide display phage libraries before biopanning with Herceptin. We have concluded that a "pre-panning" human IgG absorption will remove most non-Herceptin-specific M13 display phage mimotopes that are bound by human antibodies commonly found in healthy, human sera. Biopanning for Herceptin-specific mimotope display phage was repeated using the immunoabsorbed phage libraries. Several of the resulting mimotope phage solutions that showed high Herceptin binding affinity in direct immunoblot screening assays were used to immunize Balb/c mice. Competitive inhibition assays with Herceptin indicate that at least two mimotope phage preparations induced in mice the production of ErbB-2 binding antibodies specific to the Herceptin binding epitope. Whether or not immunization with these selected mimotope phage antigens will provide protection against transplanted ErbB-2-postive tumors in mice will be determined in a future study.

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Appendices
None